Improved Transporters and Their Uses

CROSS-REFERENCES TO RELATED APPLICATIONS

[01] NOT APPLICABLE

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[02] This invention was made with Government support under Grant No. IBN 0110622, awarded by the National Science Foundation. The government has certain rights in this invention.

FIELD OF THE INVENTION

[03] The present invention is generally related to plant genetic engineering. In particular, the invention is directed to nucleic acids and methods for conferring salt tolerance on plants and other organisms.

BACKGROUND OF THE INVENTION

[04] Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, which are predominantly sensitive to the presence of high concentrations of salts in the soil. Large terrestrial areas of the world are affected by levels of salt inimical to plant growth. It is estimated that 35-45% of the 279 million hectares of land under irrigation is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands, (which comprise 25% of the total land of our planet). Salinity has been an important factor in human history and in the life spans of agricultural systems. Salt impinging on agricultural soils has created instability and has frequently destroyed ancient and recent agrarian societies. The Sumerian culture faded as a power in the ancient world due to salt accumulation in the valleys of the Euphrates and Tigris rivers. Large areas of the Indian subcontinent have been rendered unproductive through salt accumulation and poor irrigation practices. In this century, other areas, including vast regions of Australia, Europe, southwest USA, the Canadian prairies and others have seen considerable declines in crop productivity.

[05] Although there is engineering technology available to combat this problem, through drainage and supply of high quality water, these measures are extremely costly. In most of the cases, due to the increased need for extensive agriculture, neither improved irrigation efficiency nor the installation of drainage systems is applicable. Moreover, in the arid and semi-arid regions of the world water evaporation exceeds precipitation. These soils are inherently high in salt and require vast amounts of irrigation to become productive. Since irrigation water contains dissolved salts and minerals, an application of water is also an application of salt that compounds the salinity problem.

[06] Increasing emphasis is being given to modify plants to fit the restrictive growing conditions imposed by salinity. If economically important crops could be manipulated and made salt resistant, this land could be farmed resulting in greater sales of seed and greater yield of useful crops. Conventional breeding for salt tolerance has been attempted for a long time. These breeding practices have been based mainly on the following strategies: a) the use of wide crosses between crop plants and their more salt-tolerant wild relatives (Rush and Epstein, *J. Amer. Hort. Sci.*, 106:699-704 (1981)), b) screening and selecting for variation within a particular phenotype (Norlyn, in *Genetic Engineering of Osmoregulation*, pp. 293-309 (1980)), c) designing new phenotypes through recurrent selection (Tal, *Plant & Soil*, 89:199-226 (1985)). The lack of success in generating tolerant varieties (given the low number of varieties released and their limited salt tolerance) (Flowers and Yeo, *Aust. J, Plant. Physiol.*, 22:875-884 (1995)) would suggest that conventional breeding practices are not enough and that in order to succeed a breeding program should include the engineering of transgenic crops (Bonhert and Jensen, *Aust. J, Plant. Physiol.*, 23:661-667 (1996)).

[07] Several biochemical pathways associated with stress tolerance have been characterized in different plants and a few of the genes involved in these processes have been identified and in some cases the possible role of proteins has been investigated in transgenic/overexpression experiments. Several compatible solutes have been proposed to play a role in osmoregulation under stress. Such compatible solutes, including carbohydrates (Tarcynski et al., *Science*, 259:508-510 (1995)), amino acids (Kishor *et al.*, *Plant Physiol.*, 108:1387-1394 (1995)) and quaternary N-compounds (Ishtani *et al.*, *Plant Mol. Biol.*, 27:307-317 (1995)) have been shown to increase osmoregulation under stress. Also, proteins that are normally expressed during seed maturation (LEAs, Late Embriogenesis Abundant proteins) have been suggested to play a role in water retention and in the protection of other proteins during stress. The overexpression of LEA in rice provided a moderate benefit to the plants

during water stress (Xu *et al.*, *Plant Physiol.*, 110:249-257 (1996), and Wu and Ho, WO 97/13843).

[08] A single gene (sod2) coding for a Na^+/H^+ antiport has been shown to confer sodium tolerance in fission yeast (Jia et al., *EMBO J.*, 11:1631-1640 (1992) and Young and Zheng, WO 91/06651), although the role of this plasma membrane-bound protein appears to be only limited to yeast. One of the main disadvantages of using this gene for transformation of plants is associated with the typical problems encountered in heterologous gene expression, i.e. incorrect folding of the gene product, targeting of the protein to the target membrane and regulation of the protein function.

[09] Na^+/H^+ antiporters with vacuolar antiport activity have been identified in red beet storage tissue and a variety of halophytic and salt-tolerant glycophtic plant species (Barkla and Pantoja, *Ann. Rev. Plant. Physiol.* 47:159 (1996), and Blumwald and Gelli, *Adv. Bot. Res.* 25:401 (1997)). More recently, a gene encoding a vacuolar Na^+/H^+ antiporter from *Arabadopsis thaliana*, designated *AtNHX1*, has been isolated (Blumwald et al., WO 99/47679). Overexpression of this gene in Arabadopsis, tomato, and canola has been shown to enhance salt tolerance in transgenic plants (Apse *et al.*, *Science*, 285:1256-1258 (1999), Zhang and Blumwald, *Nat. Biotechnol.*, 19:765-768 (2001), and Zhang *et al.*, *PNAS USA*, 98:12832-12836 (2001)).

BRIEF SUMMARY OF THE INVENTION

[10] The present invention provides methods of enhancing salt tolerance in plants by the introduction of a polynucleotide encoding a Na^+/H^+ transporter polypeptide, which when expressed confers increased salt tolerance in the plant. Each of these polypeptides have an amino acid sequence at least 80% identical to SEQ ID NO:2 and fewer than 530 amino acids.

[11] In some aspects of the invention, the method includes the introduction of a polynucleotide having a sequence consisting of SEQ ID NO: 5, 7, 9, 11, 13 or 15 to confer the salt tolerance to the plant.

[12] In some aspects of the invention the method includes the introduction of a polynucleotide encoding a polypeptide having a sequence consisting of SEQ ID NO:6, 8, 10, 12 or 14 to confer salt tolerance to the plant.

[13] In some aspects of the invention, the method includes the introduction of a polynucleotide which encodes a polypeptide of less than 500, or less than 475 amino acids in length to confer the salt tolerance to the plant.

In an alternative embodiment, the method for enhancing salt tolerance of a plant comprises introducing into the plant a polynucleotide encoding a Na+/H+ transporter polypeptide with an amino acid sequence at least 80% identical to SEQ ID NO:2 in which in the residue corresponding to the serine at position 508 in SEQ ID NO:2 is replaced by an amino acid that confers the increased salt tolerance of the Na+/H+ transporter polypeptide.

[14] In some embodiments, a neutral or polar amino acid replaces the serine at position 508 in SEQ ID NO:2.

[15] In some embodiments, the neutral or polar amino acid corresponding to the serine at position 508 in SEQ ID NO:2 is threonine, methionine, cysteine, asparagine or glutamine.

[16] In some embodiments, the purified polynucleotide sequence encoding a Na^+/H^+ transporter polypeptide conferring salt tolerance is SEQ ID NOS:3.

[17] In some embodiments, the Na^+/H^+ transporter polypeptide sequence encoded by the purified polynucleotide is SEQ ID NO:4.

[18] The present invention also provides for transgenic plants comprising a polynucleotide encoding a Na+/H+ transporter polypeptide, which when expressed confers increased salt tolerance in the plant; and wherein the transporter polypeptide comprises an amino acid sequence at least 80% identical to SEQ ID NO:2 of fewer than 530 amino acids.

[19] In some aspects of the invention, the transgenic plants comprising a Na+/H+ transporter polypeptide conferring increased salt tolerance in the plant has an amino acid sequence at least 80% identical to SEQ ID NO:2, and the residue corresponding to the serine at position 508 in SEQ ID NO:2 is replaced by an amino acid that confers the increased salt tolerance. In some aspects of the invention, the replacement amino acid is a polar or neutral amino acid, such as cysteine.

DEFINITIONS

[20] The term "plant" includes whole plants, shoot vegetative organs and/or structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (e.g., the mature ovary), seedlings, plant tissue (e.g. vascular tissue, ground tissue, and the like), cells (e.g. guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and

dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

[21] The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

[22] The phrase "heterologous sequence" refers to a sequence that originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (e.g. a genetically engineered coding sequence or an allele from a different ecotype or variety).

[23] A polynucleotide "exogenous to" an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include Agrobacterium-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T1 (e.g. in *Arabidopsis* by vacuum infiltration) or R0 (for plants regenerated from transformed cells in vitro) generation transgenic plant. Transgenic plants that arise from sexual cross or by selfing are descendants of such a plant.

[24] An "*NHX1* nucleic acid" or "*NHX1* polynucleotide sequence" of the invention is a subsequence or full length polynucleotide which, encodes a *NHX1* polypeptide and its complement, e.g. SEQ ID NOS:1, 3, 5, 7, 9, 11, 13, or 15. One example of an *NHX1* gene, from *Arabadopsis,* is *AtNHX1*. The *NHX1* gene products of the invention (e.g. mRNAs or polypeptides) are characterized by the ability to confer increased salt tolerance . A *NHX1* polynucleotide of the invention typically comprises a coding sequence of at least about 250 nucleotides to about 2000 nucleotides in length. Usually the *NHX1* nucleic acids of the invention are from about 400 to about 1600 nucleotides.

[25] In the expression of transgenes one of skill will recognize that the inserted polynucleotide sequence need not be identical and may be "substantially identical" to a sequence of the gene from which it was derived. As explained below, these variants are specifically covered by this term.

[26] In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional *NHX1* polypeptide, one of skill will recognize that because of codon degeneracy, a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the terms " *NHX1* polynucleotide sequence" or " NHX1 polynucleotide sequence". In addition, the terms specifically include those full length sequences substantially identical (determined as described below) with an *NHX1* gene sequence and that encode proteins that retain the function of the encoded proteins. Thus, in the case of the *Arabidopsis AtNHX1* gene disclosed here, the above term includes variant polynucleotide sequences which have substantial identity with the sequences disclosed here and which encode proteins capable of conferring salt tolerance on a transgenic plant comprising the sequence.

[27] Two polynucleotides or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the complementary sequence is identical to all or a portion of a reference polynucleotide sequence.

[28] Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two sequences over a segment or "comparison window" to identify and compare local regions of sequence similarity. The segment used for purposes of comparison may be at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

[29] Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis.), or by inspection. "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The

percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[30] The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 60% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described above (preferably BESTFIT) using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 40%, preferably at least 60%, more preferably at least 90%, and most preferably at least 95%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

[31] Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5° C. to about 20° C. lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 60° C.

However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[32] As used herein, a homolog of a particular *NHX1* gene (e.g., the *Arabidopsis AtNHX1* genes disclosed here) is a second gene (either in the same species or in a different species) which has a polynucleotide sequence of at least 50 contiguous nucleotides which are substantially identical (determined as described above) to a sequence in the first gene. It is believed that, in general, homologs share a common evolutionary past.

BRIEF DESCRIPTION OF THE DRAWINGS

[33] FIG. 1 shows yeast transformation assay results indicating modified *AtNHX1* increases salt tolerance.

[34] FIG. 2 shows yeast tranformation assay results showing C-terminal deletions of *AtNHX1* control Na^+/K^+ selectivity of the transporter.

[35] FIG. 3 shows yeast transformation assay results indicating modified *AtNHX1* increases Li^+ tolerance.

[36] FIG. 4 shows yeast transformation assay results indicating modified *AtNHX1* increases tolerance to osmotic stress.

[37] FIG. 5 shows the results of yeast transformation assays performed to characterize activity of *AtNHX1* N-terminus and tranmembrane domains

[38] FIG. 6 shows the sites of site-directed mutagenesis and truncation of *AtNHX1* conferring salt tolerance.

DETAILED DESCRIPTION OF THE INVENTION

[39] The present invention provides novel modified isolated nucleic acid molecules encoding proteins for the transport of sodium ions across a membrane of a cell, for example, from the cytosol of a cell into a vacuole, provide the cell with salt tolerance.

[40] *NHX1* genes encode membrane bound Na+/H+ antiport transporters that conduct Na^+ across a membrane using an electrochemical gradient of protons, generated, for example, by vacuolar H+-adenosine triphosphatase (ATPase) and H+-inorganic pyrophosphatase (PP_iase). In the present invention, mutated and truncated forms of *NHX1* containing augmented transport activity have been created. The modified gene products (proteins) allow more accumulation of sodium ions from the cytosol into the intracellular

compartments, such as the vacuole, compared with the entire protein. These genes allow for the engineering of salt tolerant plants by transformation of salt-sensitive crops overexpressing this gene.

[41] The invention also includes modified Na^+/H^+ antiporters having changed ion selectivity or ionic permeability leading to increased salt or drought or osmotic tolerance.

[42] The invention also relates to the modification of *NHX1* genes by using site-directed mutagenesis and deletions based on PCR amplification to screen the modified protein forms with increased transport activity or changed ion selectivity.

Isolation of nucleic acids of the invention

[43] Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology, Volumes 1-3, John Wiley & Sons, Inc. (1994-1998).

[44] Using the sequences provided here, the isolation of *NHX1* nucleic acids may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as flowers, and a cDNA library which contains the *NHX1* gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which *AtNHX1* genes or homologs are expressed.

[45] The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned *NHX1* gene or fragment thereof disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous

genes in the same or different plant species. Alternatively, antibodies raised against a *NHX1* polypeptide or fragment thereof can be used to screen a mRNA expression library.

[46] Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of the *NHX1* genes directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other in vitro amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR see PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gleaned, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990).

[47] Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. *See, e.g.*, Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.*, 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

[48] *NHX1* nucleic acids of interest may also be identified by searching nucleic acid databases, e.g., EST databases and identifying sequences with high similarity to a known *NHX1* nucleic acid sequence. Once a candidate *NHX1* nucleic acid or polynucleotide sequence of the invention has been identified, standard methods can be used to determine if the putative nucleic acid is a *NHX1* nucleic acid of the invention. Methods of assaying for *NHX1* activity are known in the art, e.g., see example 1 and Apse *et al.*, *Science.* 285, 1256-1258.

Preparation of recombinant vectors

[49] To use isolated sequences for transformation and other molecular biological techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising *et al. Ann. Rev. Genet.*, 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

[50] For example, for overexpression, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumafaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such genes include for example, ACT11 from *Arabidopsis* (Huang *et al. Plant Mol. Biol.*, 33:125-139 (1996)), Cat3 from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.*, 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al. Plant Physiol.*, 104:1167-1176 (1994)), GPc1 from maize (GenBank No. X15596, Martinez *et al. J. Mol. Biol*, 208:551-565 (1989)), and Gpc2 from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.*, 33:97-112 (1997)).

[51] Alternatively, the plant promoter may direct expression of the *NHX1* nucleic acid in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters, organ-specific promoters) or may be otherwise under more precise environmental or developmental control (*i.e.* inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of light, or sprayed with chemicals/hormones. One of skill will recognize that an organ-specific promoter may drive expression of operably linked sequences in organs other than the target organ. Thus, as used herein an organ-specific promoter is one that drives expression preferentially in the target organ, but may also lead to some expression in other organs as well.

[52] A number of tissue-specific promoters can also be used in the invention. For instance, root promoters that direct expression of root tissue nucleic acids are of particular importance to the current invention.

[53] If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

[54] The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance,

particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

Production of transgenic plants

[55] DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using biolistics, e.g., DNA particle bombardment.

[56] Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al. Embo J.*, 3:27 17-2722 (1984). Electroporation techniques are described in Fromm *et al. Proc. Natl. Acad. Sci. USA*, 82:5824 (1985). Biolistic transformation techniques are described in Klein *et al. Nature*, 327:70-73 (1987).

[57] Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch *et al. Science*, 233:496-498 (1984), and Fraley *et al. Proc. Natl. Acad. Sci. USA*, 80:4803 (1983) and Gene Transfer to Plants, Potrykus, ed. (Springer-Verlag, Berlin 1995).

[58] Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as decreased farnesyltransferase activity. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such

regeneration techniques are described generally in Klee *et al.*, *Ann. Rev, of Plant Phys.*, 38:467-486 (1987).

[59] The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Chlamydomonas, Chlorella, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Cyrtomium, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Laminaria, Linum, Lolium, Lupinus, Lycopersicon, Macrocystis, Malus, Manihot, Majorana, Medicago, Nereocystis, Nicotiana, Olea, Oryza, Osmunda, Panieum, Pannesetum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Polypodium, Prunus, Pteridium, Raphanus, Ricinus, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Triticum, Vicia, Vitis, Vigna,* and *Zea.*

[60] One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[61] Using known procedures one of skill can screen for plants of the invention by detecting the increase or decrease of *NHX1* mRNA or protein in transgenic plants. Means for detecting and quantifying mRNAs or proteins are well known in the art, e.g., Northern Blots, Western Blots or activity assays. The plants of the invention can also be identified by detecting the desired phenotype. For instance, measuring salt tolerance or drought tolerance using methods as described below.

[62] Detection of transgenic organisms of the invention

Transformed yeast strains containing the polynucleotides of the invention can be analyzed using established molecular biology techniques as well as by monitoring growth rates using culture medium selective for a wide variety of phenotypes including salt toelrance.

[63] Alternatively, transgenic plants comprising the invention may be the subject of analysis. After preparation of the expression cassettes containing the polynucleotides of the present invention and introduction of the cassettes into a plant, the resultant transgenic plants can be assayed for the phenotypical characteristics associated with increased or decreased *NHX1* expression. For example, after introduction of the cassette into a plant, the plants are screened for the presence of the transgene and crossed to an inbred or hybrid line. Progeny plants are then screened for the presence of the transgene and self-

pollinated. Progeny from the self-pollinated plants are grown. The resultant transgenic plants can be assayed for increased salt tolerance and increased drought tolerance, and decreased sensitivity to toxins. For example, a transgenic plant can be assayed for increased salt tolerance or drought tolerance. Methods for assaying for increased salt tolerance are known and include measuring the growth rate of plants at increasing salt concentrations. Plant biomass, root/shoot ratios, and tissue ion content. Root and hypocotyl growth rates can are measured and correlated with tissue water content of plants growing at different salt concentrations. Methods for assaying increased drought tolerance are also well established and include measuring transpiration rates of transgenic plant tissues, stomatal conductance, rate of water loss in a detached leaf assay or examining leaf turgor. Transgenic plants with decreased transpiration rates, for example, have increased drought tolerance.

EXAMPLES

EXAMPLE 1:SITE-DIRECTED MUTAGENESIS AND CLONING OF TRUNCATED *ATNHX1*

[64] The full length cDNA of *AtNHX1* was obtained by RT-PCR and cloned into pCR2.1-TOPO vctor (Invitrogen). The sequence was confirmed by sequencing the full open reading frame. The full length cDNA was then subcloned into yeast vector pYPGE15 by using BamH1 and EcoR1 cloning sites. Site-directed mutagenesis was carried out by following the instruction of QuikChange Site-Directed Mutagenesis Kit (Stratagene). The primes that was used for generation SM-23 are as follows: SM-23-F, ggagacaatttgatgactgcttcatgcgacccgtc; SM-23-R, gacgggtcgcatgaagcagtcatcaaattgtctcc. For the cloning of truncated *AtNHX1*, the truncated cDNA was amplified by PCR and cloned into pYPGE15 vector. The primers for the truncated *AtNHX1* cloning are as follows: EXCH-5, agctaggatccggatctagaagaagataacaatgttgg; EXCH-DL-1, agctgaattcctagggtacaaagccacgacctc; EXCH-DL-2, agctgaattcctacaagaagccacgtatactg; EXCH-DL-3, agctgaattcctaagataacatgctcgtggtg. All sequence were verified by sequencing.

EXAMPLE 2:YEAST TRANSFORMATION AND DROP TESTS

[65] The yeast cells were transformed using lithium acetate/PEG method. Yeast cells were inoculated into drop-out liquid medium and cultured overnight at 30°C. The cells were harvested by centrifuge and resuspended into APG medium (Rodriguez-Navarro

and Ramos, *J. Bacteriol.*, 159:940-945 (1984)) and adjusted the OD600 to 1.0. Serial 10 times dilutions were made and 3 1 of the cells were loaded onto APG medium with different salt supplements, or onto YPD (1% yeast extract, 2% peptone, 2% glucose) medium with 10 mg/L hygromycin.

EXAMPLE 3: INCREASED SALT TOLERANCE CONFERRED BY MODIFIED *ATNHX1*

[66] As shown in FIG. 1, the yeast mutant strain (ena1 nhx1) lacking endogenous NHX1 Na^+/H^+ antiporter was more sensitive to NaCl as well as hygromycin than the control strain (ena1). Expression of *AtNHX1* in the yeast mutant partially recovered the mutant phenotype, indicating that *AtNHX1* functions as Na^+/H^+ antiporter to compartment Na^+ into vacuole. The modified *AtNHX1*, including SM-23, Dl-1, Dl-2, and Dl-3, conferred yeast mutant cell more tolerance to salt and hygromycin than the complete form. This result suggests that the modified *AtNHX1* have higher antiport activity and could transport more Na^+ into vacuole.

EXAMPLE 4: CHANGED ION SELECTIVITY OF MODIFIED *ATNHX1*

[67] When grown on the APG medium without NaCl, the yeast cells grew equally regardless expression of complete or modified *AtNHX1* (FIG. 2). However, the yeast cells expressing modified *AtNHX1* showed higher tolerance to NaCl than that expressing complete form of *AtNHX1*, when increasing K^+ in the medium. This result suggests that the C-terminus of *NHX1* genes control the Na^+/K^+ selectivity.

EXAMPLE 5: INCREASED LI^+ TOLERANCE CONFERRED BY MODIFIED *ATNHX1*

[68] The yeast NHX1 can transport both Na^+ and Li^+. The yeast mutant lacking NHX1 gene is more sensitive to Li+ than the control yeast strain (FIG.3). Although *AtNHX1* could rescue the Na^+ sensitive phenotype of yeast mutant (Fig. 1), *AtNHX1* was unable to restore the Li^+ sensitive phenotype of the yeast mutant as shown in figure 3, indicating that *AtNHX1* has little or no activity for Li^+ transport. Surprisingly, the yeast mutant cells expressing modified *AtNHX1* displayed Li^+ tolerance almost to the level of control yeast strain. This result provides a possibility to modify transporters capable to detoxify different ions, and to engineer plants to be able to tolerance different toxic ions when overexpressing the modified transporters.

EXAMPLE 6: ELEVATED TOLERANCE TO OSMOTIC STRESS IN YEAST EXPRESSING MODIFIED *ATNHX1*

[01] Vacuolar Na^+/H^+ antiporter can sequestrate Na^+ into vacuole and keep the turgor pressure of the cell. The regulation of turgor pressure may function to drive water flow into the cell under osmotic stress. The yeast mutant cells lacking of *NHX1* showed sensitive phenotype after adding over 1M KCl to the growth medium (FIG. 4), suggesting that *NHX1* is also important in osmotic regulation. Since Na^+ was absent in the growth medium, the osmotic regulation by *NHX1* was possibly fulfilled by transport of K^+ into vacuole. Expression of complete form of *AtNHX1* could not complement the mutant phenotype, but modified forms of *AtNHX1* rescued the mutant cells growth to the level as wild type (ena1) did, indicating that modified forms of *AtNHX1* have higher K^+ transport activity than the complete form.

[70] It is possible to use the modified forms of *NHX1* to engineer plants to be more drought tolerance. Since the modified forms of *AtNHX1* could transport more K^+ into vacuole, the higher concentration of K^+ in vacuole could drive water uptake into the cell, which would generate higher strength to force more water flow through xylem stream. Theoretically, the transgenic plants overexpressing these modified forms of *AtNHX1* could take up water more effectively, and more tolerance to drought stress.

EXAMPLE 7: MODIFIED NHX1

[71] Using the methods described previously in Examples 1 and 2, modified *NHX1* polynucleotides including 23 site mutations, 3 N-terminus deletions, and 3 C-terminus deletions of *AtNHX1* cDNA were generated (See Table 1, and FIG. 5). The modified *AtNHX1* cDNAs in yeast vector were transferred into a yeast mutant lacking endogenous Na^+/H^+ antiporter NHX1 and Na^+ pump ENA1-4. The activities and ion selectivity of the transformants were determined by testing the growth of yeast harboring distinct forms of *AtNHX1* under different growth conditions.

TABLE 1

Name	Mutation	Growth
Full	NA	++++

SM-1	Y85C/L86R	+
SM-2	D137C	+++++
SM-3	D142C	+
SM-4	D145C	++++
SM-5	D157C	++++
SM-6	D168C/E169V	++++
SM-7	E180V	++++
SM-8	D185C	+++
SM-9	H202A	+++
SM-10	H205A	++++
SM-11	H210A	++++
SM-12	H285A	++++
SM-13	H289A	++++
SM-14	N290D	++++
SM-15	H301A	++++
SM-16	R390C	++++
SM-17	G391E	++++
SM-18	R404C	++++
SM-19	H407A	++++
SM-20	R411C	++++
SM-21	H499A	++++
SM-22	D506C/D507C	+++++
SM-23	S508C	+++++
DL-1	17 aa deletion	+++++
DL-2	47 aa deletion	+++++
DL-3	84 aa deletion	+++++
NDL-1	17 aa deletion	+++++++++
NDL-2	69 aa deletion	+++
DDL-3	98 aa deletion	+++

EXAMPLE 8: CHARACTERIZATION OF ATNHX1 N-TERMINUS AND TRANSMEMBRANE DOMAINS

The functions of the free N-terminus and the first several transmembrane domains of AtNHX1 were characterized using the methods described in Examples 1 and 2 above. Three N-terminal deletion forms of AtNHX1 were created and analyzed in yeast complementation tests. The deletion of the free N-terminus of AtNHX1 (NDL-1, deletion of the first 17 amino acids) conferred yeast mutant strain (*Δena1Δnhx1*) more tolerant to hygromycin and NaCl compared with the full AtNHX1. The growth of yeast cells expressing NDL-1 was Approximately 30 times higher than that expressing full AtNHX1, indicating that the free N-terminus of AtNHX1 is a negative regulator for antiporter function in yeast cells. The deletion of the first two transmembrane domains (NDL-2) and the deletion of the first three transmembrane domains (NDL-3) abolished the AtNHX1 function on some extend. These results provide the possibility to engineer plant more tolerance to salt by overexpressing modified forms of AtNHX1 with elevated antiport activity, for instance, NDL-1.

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[72] The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

SEQUENCE LISTING

SEQ ID NO:1

Wildtype *AtNHX1*

1 atgttggatt ctctagtgtc gaaactgcct tcgttatcga catctgatca cgcttctgtg
61 gttgcgttga atctctttgt tgcacttctt tgtgcttgta ttgttcttgg tcatcttttg
121 gaagagaata gatggatgaa cgaatccatc accgccttgt tgattgggct aggcactggt
181 gttaccattt tgttgattag taaaggaaaa agctcgcatc ttctcgtctt tagtgaagat
241 cttttcttca tatatctttt gccacccatt atattcaatg cagggtttca agtaaaaaag
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421 tttgacttgg gtgattatct tgctattggt gccatatttg ctgcaacaga ttcagtatgt
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1081 tcgtttctat ctaacttagc caagaagaat caaagcgaga aaatcaactt taacatgcag
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1321 tacctattac cgcaccagaa cgccaccacg agcatgttat ctgatgacaa caccccaaaa
1381 tccatacata tccctttgtt ggaccaagac tcgttcattg agccttcagg gaaccacaat

1441 gtgcctcggc ctgacagtat acgtggcttc ttgacacggc ccactcgaac cgtgcattac
1501 tactggagac aatttgatga ctccttcatg cgacccgtct ttggaggtcg tggctttgta
1561 ccctttgttc caggttctcc aactgagaga aaccctcctg atcttagtaa ggct

SEQ ID NO:2

Wildtype AtNHX1, protein sequence

MLDSLVSKLPSLSTSDHASVVALNLFVALLCACIVLGHLLEENR
WMNESITALLIGLGTGVTILLISKGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQ
FFRNFVTIMLFGAVGTIISCTIISLGVTQFFKKLDIGTFDLGDYLAIGAIFAATDSVC
TLQVLNQDETPLLYSLVFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFHLLGNFLY
LFLLSTLLGAATGLISAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLSGIL
TVFFCGIVMSHYTWHNVTESSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWRSVS
DTPGTSIAVSSILMGLVMVGRAAFVFPLSFLSNLAKKNQSEKINFNMQVVIWWSGLM
RGAVSMALAYNKFTRAGHTDVRGNAIMITSTITVCLFSTVVFGMLTKPLISYLLPHQ
NATTSMLSDDNTPKSIHIPLLDQDSFIEPSGNHNVPRPDSIRGFLTRPTRTVHYYWRQF
DDS FMRPVFGGRGFVPFVPGSPTERNPPDLSKA

SEQ ID NO:3

SM-23, S508C, single nucleic acid change, cDNA sequence

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SEQ ID NO:4

SM-23, S508C, SINGLE AMINO ACID SUBSTITUTION, PROTEIN SEQUENCE

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TLQVLNQDETPLLYSLVFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVIKKL

YFGRHSTDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESSRITTKHTFATLSFLAETFIFLYVGM

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VPRPDSIRGFLTRPTRTVHYYWRQFDDCFMRPVFGGRGFVPFVPGSPTERNPPDLSKA

SEQ ID NO:5

DL-1, 17 amino acids deletion from C-terminus, cDNA sequence

ATGTTGGATTCTCTAGTGTCGAAACTGCCTTCGTTATCGACATCTGATCACGCTTC

TGTGGTTGCGTTGAATCTCTTTGTTGCACTTCTTTGTGCTTGTATTGTTCTTGGTCA

TCTTTTGGAAGAGAATAGATGGATGAACGAATCCATCACCGCCTTGTTGATTGGG

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SEQ ID NO:6

DL-1, 17 AMINO ACIDS DELETION FROM C-TERMINUS, PROTEIN SEQUENCE

MLDSLVSKLPSLSTSDHASVVALNLFVALLCACIVLGHLLEENRWMNESITALLIGLG
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SEQ ID NO:7

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SEQ ID NO:8

DL-2, 47 AMINO ACIDS DELETION FROM C-TERMINUS, PROTEIN SEQUENCE

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SEQ ID NO:9

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ct

SEQ ID NO:10

DL-3, 84 AMINO ACIDS DELETION FROM C-TERMINUS, PROTEIN SEQUENCE

MLDSLVSKLPSLSTSDHASVVALNLFVALLCACIVLGHLLEENRWMNESITALLIGLG

TGVTILLISKGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAVGTI

ISCTIISLGVTQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFG

EGVVNDATSVVVFNAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVI

KKLYFGRHSTDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTE

SSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWRSVSDTPGTSIAVSSILMGLVMVG

RAAFVFPLSFLSNLAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGH

TDVRGNAIMITSTITVCLFSTVVFGMLTKPLISYLLPHQNATTSMLS

SEQ ID NO:11

NDL-1 cDNA sequence

atggcttctgtggttgcgttgaatctctttgttgcacttctttgtgcttgtattgttcttggtcatcttttggaagagaa
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SEQ ID NO:12

NDL-1 protein sequence

MASVVALNLFVALLCACIVLGHLLEENRWMNESITALLIGLGTGVTILLISKGKSSHL
LVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAVGTIISCTIISLGVTQFFKK
LDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVVF
NAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVIKKLYFGRHSTDRE
VALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESSRITTKHTFATLS
FLAETFIFLYVGMDALDIDKWRSVSDTPGTSIAVSSILMGLVMVGRAAFVFPLSFLSN
LAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAIMITSTI
TVCLFSTVVFGMLTKPLISYLLPHQNATTSMLSDDNTPKSIHIPLLDQDSFIEPSGNHN
VPRPDSIRGFLTRPTRTVHYYWRQFDDSFMRPVFGGRGFVPFVPGSPTERNPPDLSKA

SEQ ID NO:13

NDL-2 cDNA sequence

atgaaaagctcgcatcttctcgtctttagtgaagatctttcttcatatatcttttgccacccattatattcaatgcagg
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gcacaatcatatctctaggtgtaacacagttctttaagaagttggacattggaacctttgacttgggtgattatcttgct
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SEQ ID NO:14
NDL-2 protein sequence

MKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAVGTIISCTIISLGV
TQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVVNDAT
SVVVFNAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVIKKLYFGRH
STDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESSRITTKHT
FATLSFLAETFIFLYVGMDALDIDKWRSVSDTPGTSIAVSSILMGLVMVGRAAFVFPL
SFLSNLAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAI
MITSTITVCLFSTVVFGMLTKPLISYLLPHQNATTSMLSDDNTPKSIHIPLLDQDSFIEPS
GNHNVPRPDSIRGFLTRPTRTVHYYWRQFDDSFMRPVFGGRGFVPFVPGSPTERNPP
DLSKA

SEQ ID NO:15
NDL-3 cDNA sequence

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gtgcattactactggagacaatttgatgactccttcatgcgacccgtctttggaggtcgtggctttgtacccttgttcc
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SEQ ID NO:16
NDL-3 protein sequence

MKKKQFFRNFVTIMLFGAVGTIISCTIISLGVTQFFKKLDIGTFDLGDYLAIGAIFAATD
SVCTLQVLNQDETPLLYSLVFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFHLLGN
FLYLFLLSTLLGAATGLISAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLS
GILTVFFCGIVMSHYTWHNVTESSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWR
SVSDTPGTSIAVSSILMGLVMVGRAAFVFPLSFLSNLAKKNQSEKINFNMQVVIWWS
GLMRGAVSMALAYNKFTRAGHTDVRGNAIMITSTITVCLFSTVVFGMLTKPLISYLL
PHQNATTSMLSDDNTPKSIHIPLLDQDSFIEPSGNHNVPRPDSIRGFLTRPTRTVHYYW
RQFDDSFMRPVFGGRGFVPFVPGSPTERNPPDLSKA